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Differential control of MHR3 promoter activity by isoforms of the ecdysone receptor and inhibitory effects of E75A and MHR3

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Abstract

MHR3 is an ecdysone-inducible transcription factor whose expression in both *Manduca sexta* epidermis and the *Manduca* GV1 cell line is induced by 20-hydroxyecdysone (20E) in vitro. There are four putative ecdysone response elements (EcRE) in the 2.6-kb flanking region of the MHR3 promoter. The most proximal, EcRE1, is necessary for activation of the promoter by 20E in the GV1 cells because the mutation of EcRE1 caused the loss of responsiveness to 20E. Previous studies [Mol. Cell. Biol. 19 (1999) 4897] showed that EcR-B1/USP-1 bound only to EcRE1 and high levels of this complex increased the 20E-induced activation, whereas the presence of high USP-2 prevented this increased activation. When we expressed EcR-A alone or in combination with USP-1 under the control of *Autographa californica* baculovirus promoter (pIE1hr), the activation of the 2.6-kb promoter by 20E was reduced by about 50%. Moreover, when EcR-A was expressed together with both EcR-B1 and USP-1, it reduced the normal activation caused by EcR-B1 and USP-1 by 50%. Gel mobility shift assays showed no binding of EcR-A/USP-1 to EcRE1. The presence of EcR-A, however, reduced the binding of EcR-B1/USP-1 by about 50%. These findings suggest that EcR-A competes with EcR-B1 for binding of USP-1, leading to a decline in activity of the promoter. In addition, E75A, another ecdysone-induced transcription factor, and MHR3 itself suppressed MHR3 promoter activity by binding to the monomeric response element (MRE2). Therefore, MHR3 can be down-regulated both by itself and by E75A.

Keywords: EcR; Ultraspiracle; 20-hydroxyecdysone; Ecdysone; *Manduca sexta*; Transcription factor; MHR3; E75

Introduction

The studies of ecdysone-induced "puffing" patterns in *Drosophila melanogaster* larvae at the outset of metamorphosis led to a model for how ecdysone activates a cascade of early genes. The early gene products activate late genes in the cascade and inactivate the early genes (Ashburner et al., 1974). When 20E binds to the ecdysone receptor (EcR)/Ultraspiracle (USP) complex, it initiates this transcriptional ecdysone cascade leading to molting and metamorphosis (Cherbas and Cherbas, 1996; Riddiford et al., 2001; Thummel and Chory, 2002). Many, but not all, of the early genes from these puff sites have been shown to be transcription factors, and many of them are members of the nuclear receptor superfamily (Mangelsdorf et al., 1995; Thummel, 1996).

In *Drosophila*, there are three isoforms of EcR (EcR-A, EcR-B1 and EcR-B2) (Talbot et al., 1993) but only one USP (Oro et al., 1990; Henrich et al., 1994), whereas the tobacco hornworm, *Manduca sexta*, has two EcRs (EcR-B1 and EcR-A) (Fujiwara et al., 1995; Jindra et al., 1996) and two USPs (USP-1 and USP-2) (Jindra et al., 1997). In *Drosophila*, most imaginal tissues express predominantly EcR-A at pupariation whereas most larval tissues express EcR-B1 (Talbot et al., 1993). EcR null mutations resulted in embryonic lethality, whereas EcR-B1 mutants have reduced viability as larvae but show major lethality at the beginning of metamorphosis (Bender et al., 1997). This phenotype suggests a dissociation of metamorphic development, with larval tissues tending to arrest and imaginal tissues progressing in their development. The USP mutant, usp^2, has a phenotype that is very similar to the EcR-B mutants (Hall and Thummel, 1998). The lack of USP causes premature neuronal differentiation in the wing even in the absence of

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20E (Schubiger and Truman, 2000), indicating that the unliganded EcR/USP complex also is suppressing early metamorphic development, at least in imaginal discs. The presence of 20E removes this inhibition, thus allowing development to occur (Schubiger and Truman, 2000).

The early gene protein products, such as E74, E75, and Broad (BR), regulate the initial cascade of responses to ecdysone in the mid to late 3rd instar larvae (Burtis et al., 1990; DiBello et al., 1991; Segraves and Hogness, 1990; Thummel, 1990). DHR3 (Koelle et al., 1992), a homolog of the retinoid orphan receptor (RORα) in vertebrates (Giguére et al., 1994), is activated later when the ecdysone titer increases; therefore, it is considered as an early–late gene (Huet et al., 1995). DHR3 is required for normal embryonic development of the central nervous system and for hatching but has little, if any, effect on cuticle formation (Carney et al., 1997). Yet in the adult, DHR3 is required for the development of bristles, wings and cuticle, but has no apparent action in eye or leg development (Lam et al., 1999). CHR3, a *C. elegans* homolog of DHR3, is an essential epidermal factor that is associated with abdominal epidermal cell function including molting and body size regulation (Kostrouchova et al., 1998).

Several genes encoding 20E-induced transcription factors have been cloned in *Manduca* such as MHR3 (Palli et al., 1992), a *Manduca* homolog of DHR3, E75A and E75B (Segraves and Woldin, 1993; Zhou et al., 1998b), Broad (BR) (Zhou et al., 1998a), E74A and E74B (Stilwell et al., 2003), MHR4 (Weller et al., 2001), and βFTZ-F1 (Weller et al., 2001). During the final larval molt, the changing ecdysteroid titer coordinates changes in both the EcR and USP isoforms as well as in the transcription factor cascades (Riddiford et al., 1999) along with EcR-B1, EcR-A, USP-1 and USP-2. When the ecdysteroid titer increases, both EcR-B1 and USP-2 increase and E75A mRNA transiently appears. Both EcR-B1 and E75A then decline sharply at the peak ecdysteroid titer when MHR3, EcR-A and E75B appear (Zhou et al., 1998b). MHR3 appears when the ecdysone titer peaks (Palli et al., 1992; Langelan et al., 2000).

20E stimulates MHR3 expression in the general abdominal epidermis (Palli et al., 1992), and MHR3 protein appears in a pattern-specific manner as the 20E titer rises during the larval molt. The crochet epidermis is more sensitive to 20E for the expression of MHR3 than the abdominal epidermis where MHR3 protein is sequentially expressed by the spiracle, the dorsal, and the trichogen and tormogen cells (Langelan et al., 2000). In both the *Manduca* GV1 cell line and *Manduca* larval epidermis, 20E induces MHR3 with similar kinetics (Palli et al., 1992; Lan et al., 1997). This induction was found to require EcR-B1 and USP-1, but to be inhibited by USP-2 (Lan et al., 1999).

Here we show that EcR-A, like USP-2, prevents the activation action of the EcR-B1/USP-1 on the MHR3 promoter. In addition, E75A, which appears shortly before MHR3 expression in the epidermis, suppresses the MHR3 expression and MHR3 itself suppresses its own gene.

Materials and methods

Cell line, animals, and hormone treatment

The *Manduca* GV1 cell line was maintained with 10% fetal bovine serum as previously described (Lan et al., 1997). Larvae of the tobacco hornworm, *M. sexta*, were reared on an artificial diet (Bell and Joachim, 1976) at 25.5°C under 12L:12D conditions. Lights-off was designated as 00:00 Arbitrary Zeitgeber Time (AZT) (Pittendrigh, 1965).

The concentration of 20-hydroxyecdysone (20E) (a gift of Dr. Takeshi Matsumoto) was determined spectrophotometrically (ε_{240} = 12,677 in EtOH). Anisomycin (AMC) (Sigma, St. Louis, MO) was used to inhibit protein synthesis at 10 μg/ml, a concentration that inhibited >99% total protein synthesis in the cultured epidermis (Hiruma et al., 1995). The solution was prepared shortly before its addition.

Construction of plasmids

The expression vectors for pIE1^{hr}/EcR-B1, pIE1^{hr}/USP-1, and pIE1^{hr}/USP-2 using the promoter of the immediate early gene of the *Autographa californica* baculovirus were used in our previous studies (Lan et al., 1999). The cDNAs of E75A (Zhou et al., 1998b) and MHR3 (Palli et al., 1992) were ligated into the pIE1hr/PA vector (a gift from Paul D. Friesen); the pIE1^{hr}/E75A was a gift from Dr. Que Lan. Both cDNAs were shown to produce their proteins using the TNT Coupled Reticulocyte Lysate System (Promega).

To remove the Aβ region from the *Manduca* EcR-A cDNA, we used a PCR method with the following primers. 5′-TTCGCGGATCCGCCACCATGAACCAACA-GATCTGT-3′ and 5′-CCCATCAGTACTACATCCATT-TATACTTGAGGCCGGTGATAATTCTTCTCGACC-TATTTTCCCATTTGG-3′. Amplification using *Pfu Turbo* DNA polymerase (Stratagene) occurred with denaturing temperature of 95°C for 2 min, annealing of 55°C for 1 min, and extensions of 72°C for 1 min. The original EcR-A cDNA in the CMX-Not plasmid (Jindra et al., 1996) was used as template. The resulting product was digested with *Bam*HI and *Sca*I, and ligated into the *Bam*HI/*Sca*I digested template plasmid. The removal of the Aβ region was confirmed by sequencing. The cDNA without the Aβ region was ligated to the pIE1^{hr}/PA vector.

Mutation of MHR3 promoter

To mutate EcRE1, a PCR approach was used, and the PCR primer sequences are as follows: 5′-CATTCCCCCGGGTA-GAAAGCACCGGTTGACGCCGGCGCGTC-3′ and 5′-TTTGGGTCCCGGGTAGAAAGC-3′. Amplification was performed as above using the −1216 MHR3 CAT construct as template. The PCR product was digested with *Sma*I and *Mlu*I, and ligated to the *Sma*I/*Mlu*I digested MHR3 construct to replace the fragment. The mutation was confirmed by sequencing.

RNAi production

To produce EcR-B1-specific RNAi, two EcR-B1-specific regions were amplified. The forward primers were 5′-ACGAAACTGCAGTTATGGCGAGGAAAAAGTGAA-3′ and 5′-ACGAAACTGCAGATGAGACGCCGCTGGT-CAAAC-3′, and the reverse primer was 5′-TGTTCCAA-GCTTGACGACATGGACTCGTTCTC-3′. The two regions were amplified separately under the same PCR conditions mentioned above except for 1-min denaturation when EcR-B1 cDNA (Fujiwara et al., 1995) was used as template. The products were digested with *Pst*I and *Hin*dIII and they were ligated into the *Hin*dIII-digested pIE1^{hr}/PA vector, so that an EcR-B1-specific region formed double-stranded RNA when the construct was transfected into the *Manduca* cell line.

Transient transfection and CAT assays

All the transfections were performed as previously described using Lipofectin reagent (Lan and Riddiford, 1997). An hsp 70-β-galactosidase plasmid (pXH70ZT) was cotransfected in all experiments for normalization of the transfection efficiency. CAT protein assays were done using CAT ELISA kit (Roche Molecular Biochemicals).

RNA extractions and immunoblots

Total RNA from the cells was isolated using the modified method of Chomczynski and Sacchi (1987) (Hiruma et al., 1997). Dot blot hybridizations (5 μg total RNA as determined spectrophotometrically per dot) were performed as described (Hiruma and Riddiford, 1990). The cDNA probes and the hybridization conditions were as described in Palli et al. (1992) for MHR3, Zhou et al. (1998b) for E75A, and Hiruma et al. (1999) for EcR-A, EcR-B1, USP-1, and USP-2. The hybridized filters were analyzed by the molecular imager, Bio-Rad Model GS-505.

Cellular proteins were extracted in the lysis buffer (Roche Molecular Biochemicals) (10 mM, MOPS, 10 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5 mM PMSF, 1 μg/ml antipain, pH 6.5). Protein concentration was determined with the BCA protein assay kit (Pierce), and 12 μg total protein were separated by 9% SDS polyacrylamide gel. Immunoblotting and the detection of protein were performed as described (Hiruma et al., 1999).

Gel mobility shift assays

The oligonucleotides used as probes (EcRE1, 5′-CGGGGTCAATGAACCGGT-3′; EcRE2, 5′-GAACGTT-GATTGCGCAAA-3′; EcRE3, 5′-TATCGTTGACAACT-CATT-3′; EcRE4, 5′-GTAGTGACAATGACCAGCA-3′, monomeric receptor element 1 [MRE1], 5′-TTTCC-GGGGTCAACGACC-3′, MRE2, 5′-GCAATTGTAT-TAAGGTGAAGGTCATTCTCCAGT-3′) were synthesized by Gibco BRL or Invitrogen. The probes were labeled as described previously (Hiruma et al., 1995). One strand of the oligonucleotide was ^{32}P-labeled at the 5′ end with T4 polynucleotide kinase and [γ-^{32}P]ATP, 10-fold molar excess of the cold complementary strand was added, and the mixture was heated at 95°C for 2 min, followed by a slow cooling to the room temperature. The double-stranded labeled probes were purified by Sephadex G-50 columns (Sambrook et al., 1989). The cold nucleotides used for competitors were prepared as described in Hiruma et al. (1995).

The preparations of proteins for the gel mobility shift assays (GMSA) were as follows. The GV1 cell extracts constitutively expressing EcR-B1, EcR-A, USP-1, USP-2, E75A, and MHR3 were prepared as described in Lan et al. (1999). In vitro-transcribed-translated EcR-B1, EcR-A, USP-1, and USP-2 were prepared with the TNT Coupled Reticulocyte Lysate system (Promega).

Five micrograms of cell extract or 2 μl of reticulocyte lysate for each of the tested translation products in 19 μl of buffer containing 1 μg of poly(dI–dC)(dI–dC) (Sigma), 12 mM HEPES (pH 7.9), 60 mM KCl, 65 mM NaCl, 7.5 mM $MgCl_2$, 6.6 mM EDTA, 1.2 mM DTT, and 12% glycerol were preincubated on ice for 5–10 min, and then 50 fmol of labeled probe and competitor DNA were added. The reaction mixture was incubated for 20 min at 0°C. The mixture was then run on a 4% polyacrylamide gel containing 2.5% glycerol in 1× TBE (45 mM Tris–borate, 1 mM EDTA), which had been pre-run for 3–4 h in 1× TBE. The gels were dried and then analyzed by the Molecular Imager System, model GS-505 (Bio-Rad).

Results

Construction of a translatable EcR-A cDNA

The *Manduca* EcR-A cDNA (Jindra et al., 1996) could not produce a transcript that could be translated into protein despite several modifications including the addition of the Kozak consensus sequence (Kozak, 1991) before the start site. Also, rescreening of our existing cDNA libraries yielded no other cDNA (unpublished). At its 3′ end, the *Manduca* EcR-A-specific region contains an exon (Aβ) encoding 43 amino acids (Jindra et al., 1996) that is not found in either *Bombyx* (Kamimura et al., 1997) or *Drosophila* (Talbot et al., 1993) EcR-A cDNAs. In *Manduca*, about half of the individuals of our colony lack this exon (Jindra et al., 1996). Therefore, we removed this region by the method described in Materials and methods.

In vitro transcription/translation assays showed that the cDNA without the Aβ region produced EcR-A protein (data not shown). This cDNA was ligated into the pIE1^{hr}/PA plasmid (pIE1^{hr}/EcR-A) and was transfected into GV1 cells. These cells then produced EcR-A protein as detected by immunoblot analysis with the EcR-common antibody

(Jindra et al., 1996) (Fig. 1). The molecular weight of EcR-A (~70 kDa) was slightly lower than that of EcR-B1 produced by the transfected pIE1^{hr}/EcR-B1 as expected due to the smaller size of the EcR-A-specific region (Fujiwara et al., 1995; Jindra et al., 1996). Fig. 1 also shows that no EcR-A is detectable in the nontransfected GV1 cells.

Hormonal regulation of EcR, USP, MHR3, and E75A in the GV1 cells

EcR and USP

When GV1 cells were treated with 2 μg/ml 20E, mRNAs of EcR-B1 and USP-2 increased about twofold (Fig. 2). The EcR-B1 mRNA peaked at 6 h, then declined. USP-2 mRNA increased initially, then plateaued between 6 and 24 h. EcR-A and USP-1 mRNA levels showed little significant change during the 24-h culture period with 20E. The maximal EcR-B1 level expressed in the GV1 cells was very similar to that found in the highest amount in epidermis on day 1 wandering (Jindra et al., 1996), but those of EcR-A and USP-1 were about half that in the epidermis, and that of USP-2 was slightly lower at times of high expression (Jindra et al., 1996, 1997; Hiruma et al., 1999).

MHR3 and E75A

Neither E75A nor MHR3 mRNA was detected in the absence of 20E (Fig. 2). The E75A gene was rapidly activated by 2 μg/ml 20E. The mRNA was detected 0.5–1 h after exposure to 20E, peaked at 4 h, then declined slowly (Fig. 2). Yet the peak level of E75A RNA in these GV1 cells was less than 10% of that found in the epidermal cells during the pupal molt (W2) (Zhou et al., 1998b).

When the cells were cultured with 2 μg/ml 20E, MHR3 mRNA appeared 2–3 h later, then attained a plateau between 6 and 12 h exposure to 20E (Fig. 2) as previously found by Lan et al. (1997). The level of induced mRNA was equivalent to that expressed in the epidermis 6 h before head capsule slippage stage (HCS) during the 4th instar molt (Langelan et al., 2000). The addition of the protein synthesis inhibitor, anisomycin (AMC), which prevented > 99% protein synthesis in the epidermis under our culture conditions (Hiruma et al., 1995), allowed about 50% of the normal MHR3 mRNA accumulation, indicating that protein synthesis is required for its full activation as is also found in the cultured epidermis (Palli et al., 1992).

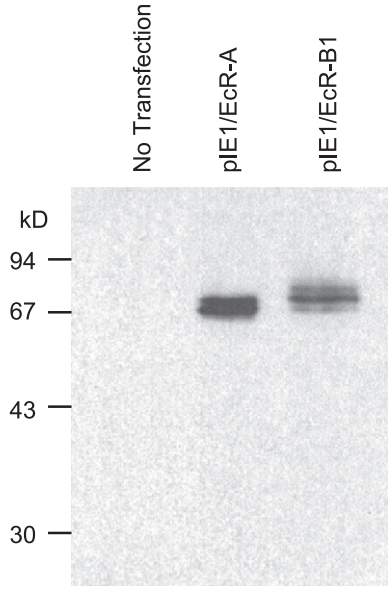

Fig. 1. Expression of EcR-A in GV1 cells. The EcR-A cDNA inserted into the pIE1^{hr}/PA vector (pIE1^{hr}/EcR-A) and pIE1^{hr}/EcR-B1 was transfected into the GV1 cell line and 15 μg cell extracts were run on a 9% polyacrylamide gel, and EcR proteins were detected with EcR-common antibody, 9B9 (Jindra et al., 1996).

Roles of EcR-B1, EcR-A, USP-1, and USP-2 on the regulation of the MHR3 promoter

The −2571 MHR3 promoter contains four putative EcREs and is activated in transfected GV1 cells 1–3 h after the addition of 20E with high levels by 6 h (Lan et al., 1999). Therefore, in this paper, we have used a 6-h culture regime to study the activation and inhibition of this promoter by various factors. The activity level of the −2571 construct cultured with 2 μg/ml 20E for 6 h was referred as 100 for all the further experiments.

The −2571 and −1216 constructs

Fig. 3A shows that when the construct was transfected into the GV1 cells, the −2571 promoter was activated in response to 20E for 6 h as found by Lan et al. (1999). When both EcR-B1 and USP-1 were expressed at constitutively high levels by cotransfecting both pIE1^{hr}/EcR-B1 and pIE1^{hr}/USP-1 constructs, the activation of the promoter by 20E was about twofold higher. Expression of either EcR-B1 or USP-1 by itself had little effect. These results are consistent with our previous results (Lan et al., 1999). When either USP-2 or EcR-A was expressed together with EcR-B1/USP-1, the increased activation was suppressed (Fig. 3A). The suppressive activity of USP-2 was stronger than that of EcR-A. Whenever USP-2 or EcR-A or both were expressed in the absence of additional EcR-B1, the levels of the promoter activity declined to below the control (None) level (Fig. 3A).

When EcRE4 and its adjacent region were removed (−1216 construct), the activation of the promoter by 20E was reduced by about 50% (Fig. 3B), but the expression of EcR-B1/USP-1 still induced a twofold higher activation. This result indicates that EcRE4 and its flanking region are important to enhance the promoter activity. The suppression of the promoter activity by USP-2 and EcR-A mirrored that found for the −2571 construct.

The −596 and −515 constructs

Further truncation deleting EcRE3 (−596) reduced activation to about 20%, but the removal of EcRE2 (−515) had

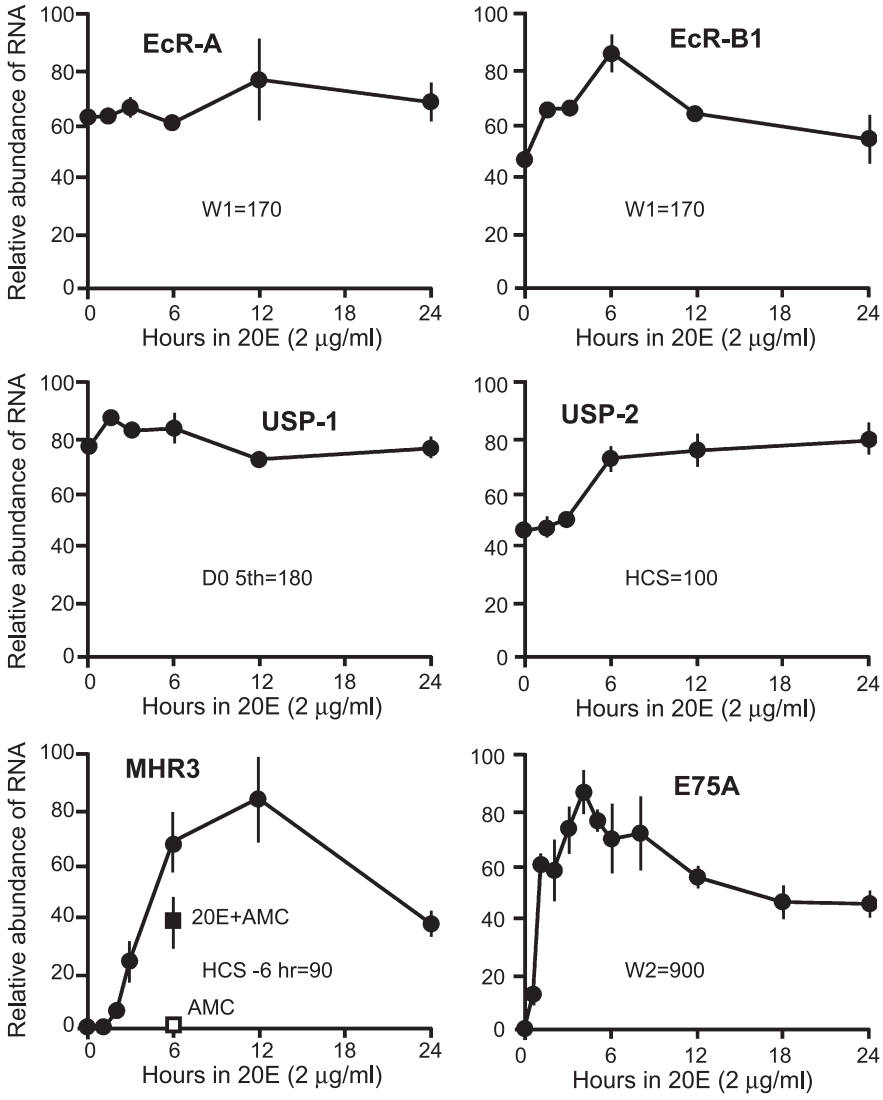

Fig. 2. Hormonal regulation of the RNA expressions of EcR, USP, MHR3, and E75A in GV1 cells. The cells were treated with 2 µg/ml 20E for varying hours, and then total RNAs were isolated to determine the amount of RNA by dot blot hybridizations. $N = 6$. The relative abundance of RNAs of EcR-A, EcR-B1, USP-1, USP-2, MHR3, and E75A were referred to the levels of mRNA in the epidermis as Day 1 Wandering stage (W1) = 170, W1 = 70, Day 0 5th = 180, HCS = 100, 6 h before HCS = 90 and W2 = 900, respectively.

no additional effect (Fig. 3C). Thus, EcRE3 and its surrounding regions are also necessary for the enhanced activation, but EcRE2 is not. Both constructs showed over twofold increased activation in response to 20E when increased levels of EcR-B1 and USP-1 were present. Just as with the longer constructs (−2571 and −1216), expression of EcR-A reduced this activation.

The mutation of EcRE1

Fig. 3C clearly shows that the promoter containing only EcRE1 responded to 20E and that constitutively high levels of EcR-B1 and USP-1 enhanced this response. Lan et al. (1999) showed that EcRE1 was crucial for the activation of the MHR3 promoter, because the removal of the 95 bp (nt −342 to −247; *Sma*I/*Bam*HI) including EcRE1 completely abolished the promoter activity. When EcRE1, GGGGTCAATGAACC, was mutated to GGG**TAG**AA**A**G **C**ACC in the −1216 construct, the basal activity increased but there was little response to 20E (Fig. 4). The gel mobility shift assays showed that both reticulocyte lysate- and GV1-produced EcR-B1/USP-1 did not bind to this mutated element (data not shown), supporting the inactivation.

These results clearly show that EcRE1 is essential for the activation of the MHR3 promoter by 20E. EcRE4 and EcRE3 and/or their flanking regions are necessary for full activation. EcR-B1/USP-1 activates the promoter by binding to EcRE1 in the presence of 20E, unless EcR-A and/or USP-2 are present.

Action of EcR-A in the absence of EcR-B1

All the experiments conducted above were performed in the presence of EcR-B1, as EcR-B1 expression is induced

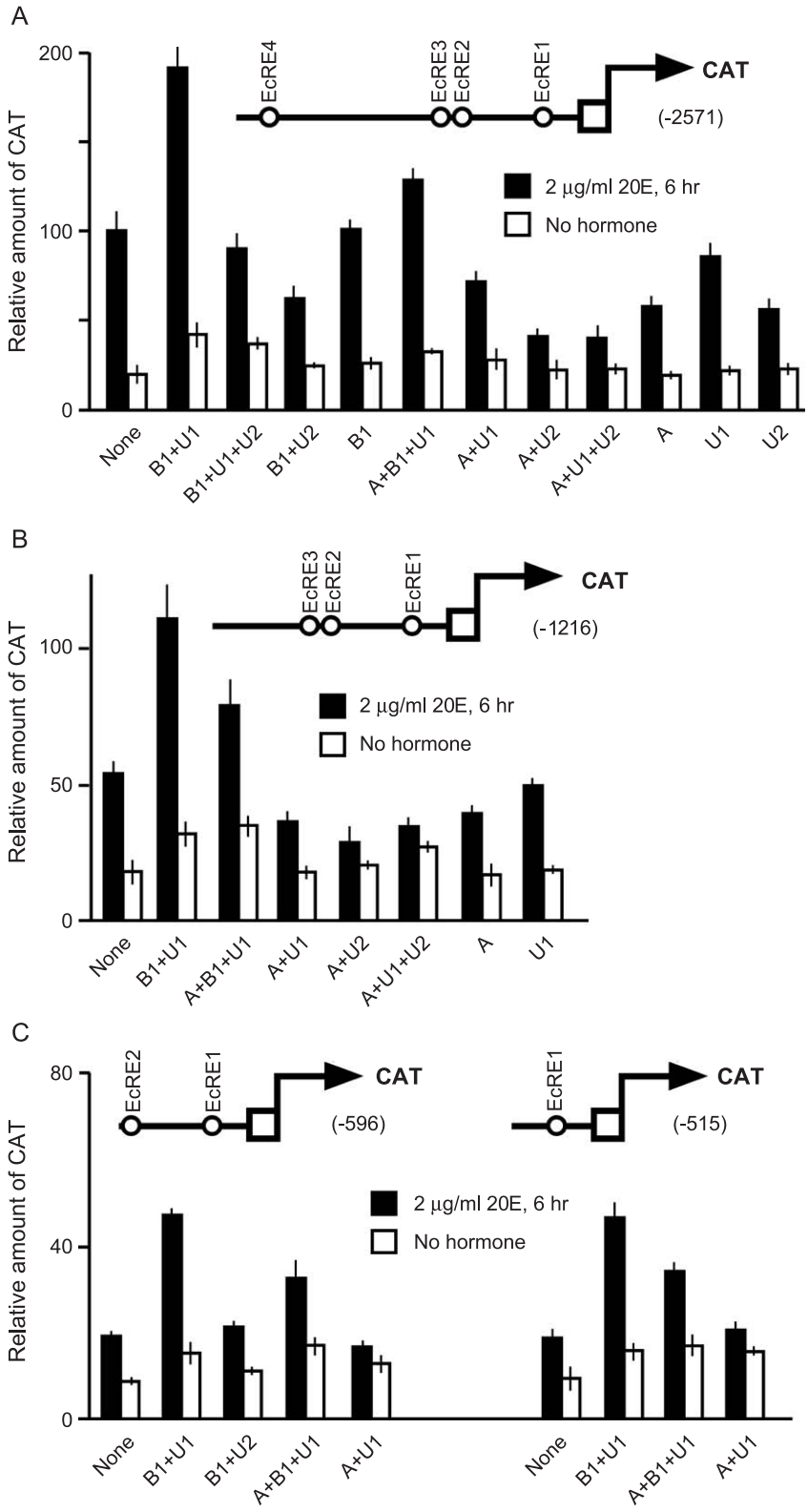

Fig. 3. Effect of EcR and USP isoforms on activation and inactivation of the MHR3 promoter. The pIE1^{hr} expression vectors for EcR-B1, EcR-A, USP-1, and USP-2 were transfected with the MHR3 promoter ligated to a CAT cDNA, and the cells were exposed to 2 μg/ml 20E for 6 h. (A) −2571 bp MHR3 promoter construct, $N = 15–20$. (B) −1216 bp, $N = 15–20$. (C) −596 and −515 bp constructs, $N = 10–15$. None, only empty pIE1^{hr} plasmid was transfected; B1, EcR-B1; A, EcR-A; U1, USP-1; U2, USP-2. ■, 20E; □, no hormone.

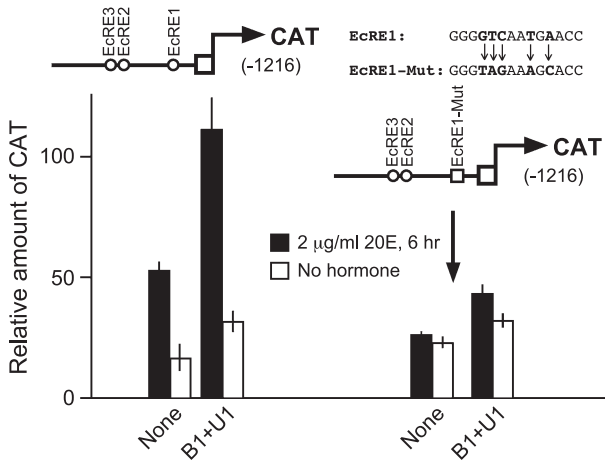

Fig. 4. Reduction of 20E-induced promoter activity by the mutation of EcRE1 in the −1216 promoter. EcRE1, GGGTCAATGAACC, was mutated to GGGTAGAAAGCACC. $N = 7$. The cells were cultured as in Fig. 3. The abbreviations were as in Fig. 3. ■, 20E; □, no hormone.

by 20E in the GV1 cells (Lan et al., 1999; Figs. 1 and 5A). To prevent the expression of EcR-B1, we transfected a construct that expresses both the sense and the antisense RNA of the EcR-B1-specific region (see Materials and methods), thus forming double-stranded RNA in the cells. Transfection of EcR-B1-specific RNAi prevented the normal increase in EcR-B1 protein upon exposure to 20E for 6 h (Fig. 5A). As our transfection efficiency is about 40% (Lan and Riddiford, 1997), the overall reduction of EcR-B1 protein level was quite high. Fig. 5B shows that the basal activity of the promoter was increased by the expression of EcR-B1 RNAi, but promoter activation by 20E was severely reduced (an activation ratio of 8X to 1.3×). Under these conditions, EcR-A and EcR-A/USP-1 had little effect.

Binding of EcR and USP to the putative EcREs

Gel mobility shift assays (GMSA) showed that an EcR-B1/USP-1 complex bound to EcRE1, but not to either EcRE2 or EcRE3, and that USP-2 almost completely prevented this binding (Lan et al., 1999; data not shown). They also showed that protein(s) bound to EcRE3 in a probe-specific fashion, but little protein bound to EcRE2.

When GMSA were performed with EcR-A using GV1 cell extracts, no binding to EcRE1 was detected, irrespective of the presence or absence of USP-1 or USP-2 (Fig. 6A). Yet EcR-A reduced the binding of an EcR-B1/USP-1 complex to EcRE1 about 50%, but it never completely prevented this binding as previously found for USP-2 (Lan et al., 1999). These results are consistent with those of the functional transfection assays in which USP-2 was a stronger suppressor than EcR-A (Fig. 3A). In addition, there was no binding of EcR-A/USP-1, EcR-A/USP-2, or EcR-A to EcRE2, EcRE3 or EcRE4. Also, the EcR-B1/USP-1 complex did not bind to EcRE4 (data not shown).

When reticulocyte lysate-produced proteins, instead of cell extracts, were used, both EcR-A/USP-1 and EcR-A/USP-2 complexes bound to EcRE1 as did the EcR-B1/USP-1 complex (Fig. 6B). Importantly, the addition of a GV1 cell extract prevented binding of the EcR-A/USP complexes.

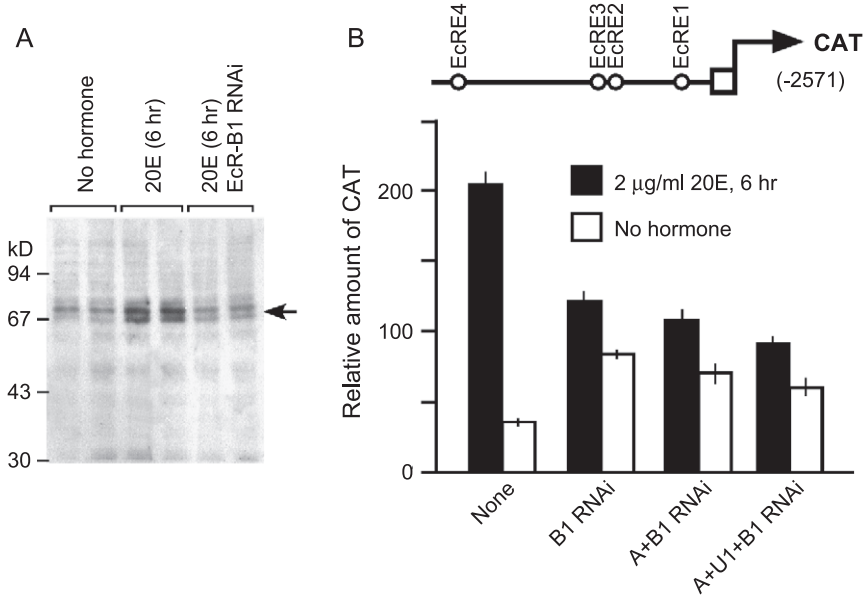

Fig. 5. (A) Prevention of EcR-B1 expression by the EcR-B1-specific RNAi transfection. The pIE1^{hr} construct that produces sense and antisense RNAs of EcR-B1-specific region was transfected to the cells for 48 h, then the cells were treated with 2 μg/ml 20E for 6 h followed by immunoblot. The EcR-B1-specific monoclonal antibody (Jindra et al., 1996) was used. The arrow indicates EcR-B1. (B) Inhibition of the activation of the MHR3 promoter (−2571 construct) by EcR-B1-specific RNAi transfection, and the action of EcR-A and USP-1 on the promoter activity. The cells were cultured with 2 μg/ml 20E for 6 h. The abbreviations were as in Fig. 3. $N = 4–6$. ■, 20E; □, no hormone.

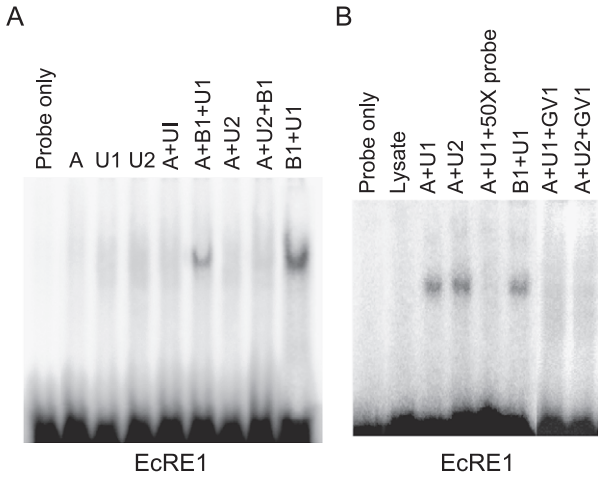

Fig. 6. Gel mobility shift assays using EcRE1. (A) GV1 cell extracts expressed denoted factors were used. (B) TNT-coupled reticulocyte lysate produced proteins were used. The abbreviations were as in Fig. 3. GV1, 5 μg GV1 cell extract was added.

E75A and MHR3 suppress the activation of the MHR3 promoter

In the 2571-bp promoter region of MHR3, there are at least two putative monomeric response elements (MRE1 and MRE2) (Fig. 7) (Harding and Lazar, 1993). One of them, MRE2, is preceded by an AT-rich region (TAT-TAAGGTGA**AGGTCA**TTC) that has been shown to be important for the monomeric binding of nuclear receptors (Harding and Lazar, 1993). By contrast, MRE1 is not preceded by these sequences. Both E75A and DHR3, the *Drosophila* ortholog of MHR3, in *Drosophila* bind to the consensus Ear 1 (A/T-AGGTCA) (Horner et al., 1995).

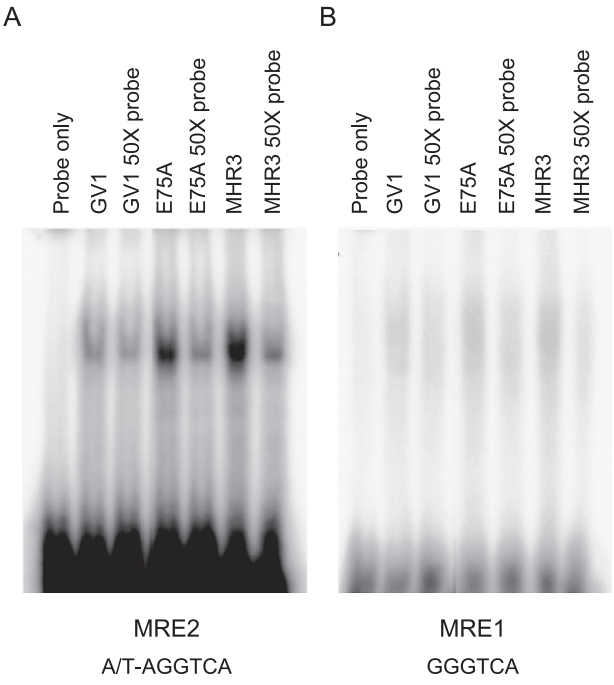

Fig. 8. Gel mobility shift assays using MRE2 and MRE1. GV1 cell extracts produced E75A and MHR3 were used as protein source.

When either E75A or MHR3 was expressed constitutively at high levels in the GV1 cells, the activation of the −2571 MHR3 promoter construct by 20E was reduced by about 50% (Fig. 7). When the promoter was truncated to remove MRE2, neither MHR3 nor E75A was able to inhibit the 20E induction (construct −1216, −596, and −515 in Fig. 7). GMSA showed that both MHR3 and E75A bound to MRE2, but not to MRE1 (Fig. 8). Neither E75A nor MHR3 bound to any of the four EcREs in the promoter region (data not shown).

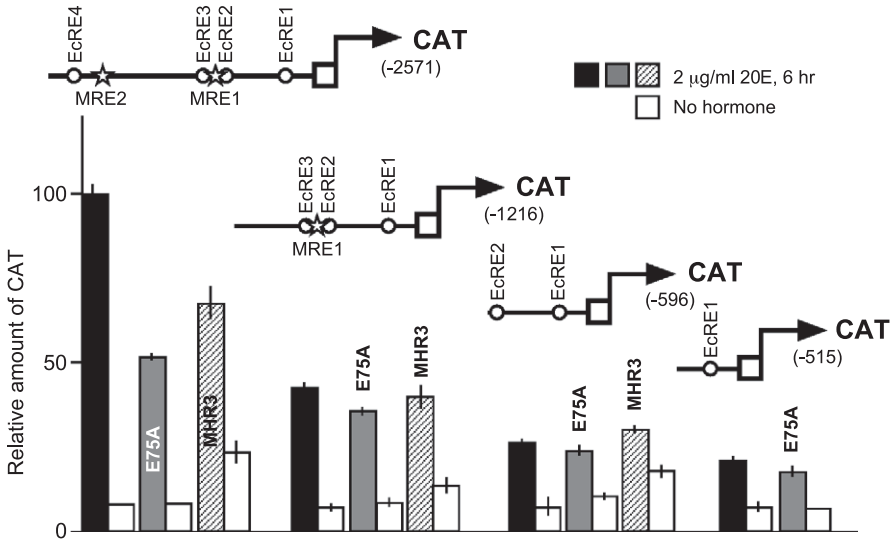

Fig. 7. Suppression of the MHR3 promoter by E75A and MHR3. pIEh^{hr}/E75A and pIEh^{hr}/MHR3 were transfected and cultured with 2 μg/ml 20E for 6 h. ■, 20E; □, no hormone. −2571, −1216, and −596 constructs, $N = 15$; −515 construct, $N = 5$.

Discussion

MHR3 expression in both *Manduca* epidermis and the *Manduca* GV1 cell line is induced by 20E. The patterns of MHR3 RNA expression are essentially the same in both cells. Addition of a protein synthesis inhibitor reduced the MHR3 RNA accumulation by about half in both cell types (Palli et al., 1992; Fig. 2), indicating that 20E acts directly on the gene, but some protein is required for its full activation. Therefore, the GV1 cells provide a good system in which to study the regulation of the MHR3 promoter by various 20E-regulated transcription factors.

EcRE1 is the primary EcR/USP binding site

There are four putative ecdysone response elements (EcRE) in the 2.6-kb flanking region of the MHR3 promoter, but only the most proximal EcRE1 apparently is necessary for activation of the promoter by 20E (Lan et al., 1999; Fig. 6). When this element and its flanking region (95 bp) was removed (Lan et al., 1999) or when this element was mutated (Fig. 4), no activation occurred.

Full activation by 20E requires the presence of the upstream promoter containing both EcRE4 and EcRE3 as removal of this region caused about an 80% reduction in activation with 50% of the reduction occurring with the truncation to −1216 bp (Lan et al., 1999; Fig. 3). Removal of the region containing EcRE2 had no effect. Some cellular protein(s), but not EcR or USP, bound to EcRE3 (Lan et al., 1999), but no proteins bound to EcRE4 (data not shown). The proteins binding to EcRE3, however, are neither E75A nor MHR3. These results suggest that EcRE3, but not EcRE2 or EcRE4, may be important for full activation as well as some unknown response element(s) in the upstream region.

Suppression of activation of the MHR3 promoter by EcR-A

Activation of the MHR3 promoter requires EcR-B1 because when EcR-B1 levels were decreased by use of EcR-B1 RNAi, very little activation occurred (Fig. 5). Addition of EcR-A could not substitute for EcR-B1 under these conditions. Nor could EcR-A in the presence of either USP-1 or USP-2 activate the promoter. These findings are similar to those of Hu et al. (2003) who found that the isoform-specific domains of *Drosophila* EcR-B1 and -B2 could activate the *eip71* promoter but that of EcR-A could not. Using an artificial promoter containing four copies of a synthetic EcRE (PAL1), Mouillet et al. (2001) found that both EcR-B isoforms activated whereas activation by EcR-A was only about one-third that of EcR-B1. When they used only the isoform-specific domains fused to a Gal4 DNA-binding domain, the EcR-A-specific domain showed no activation and reduced basal promoter activity.

Our gel shift experiments suggest that the inability of EcR-A to activate the MHR3 promoter in the GV1 cells is due to its inability to bind to EcRE1. This inability to bind is not an intrinsic feature of EcR-A as EcR-A produced in reticulocyte lysates can bind as a heterodimer with either USP-1 or USP-2 to this EcRE. Addition of cell extract, however, prevented this binding. We do not know whether this effect is due to a modification of EcR-A by the extract or to factors that interact with it and suppress the binding. Importantly, this effect is specific to EcR-A.

Despite its lack of binding to the ecdysone response element, EcR-A reduced the binding of EcR-B1/USP-1 to EcRE1 by about 50% and thereby partially inhibited its activation of the MHR3 promoter. Because this reduction cannot be a competition with EcR-B1 for binding, EcR-A likely competes for binding to USP-1 and thus reduces the amount of EcR-B1/USP-1 complex available to bind to EcRE1. Consequently, activation of the promoter by 20E is less. The same explanation presumably holds for USP-2 competing with USP-1 for binding to EcR-B1. A comparison of the nearly total reduction of binding by high expression of USP-2 (Lan et al., 1999) versus the partial reduction by high expression of EcR-A suggests that USP-2 is more effective in competition for EcR-B1 than EcR-A is for USP-1. The reason for the difference in competitiveness is unclear. One possibility is the amount produced by the pIE promoter is different in the two cases. When EcR-A was overexpressed in *Drosophila* wing discs using an *engrailed*-Gal 4 driver, both DHR3 and E75B expression was decreased (Schubiger et al., 2003). This result was thought to be due to the competition of EcR-A with EcR-B1 for binding sites leading to the reduction of activation since EcR-A had been found to be a poor activator (Mouillet et al., 2001; Hu et al., 2003), but it could be equally as well explained by EcR-A competing for USP.

E75A as a repressor of MHR3 and a feedback action of MHR3

E75A is an ecdysone-inducible transcription factor (Segraves and Hogness, 1990; Zhou et al., 1998b). In the GV1 cells, E75A expression was also induced by 20E, but the amount induced was extremely low when compared with expressing tissues such as epidermis (Fig. 2). In *Manduca* epidermis, E75A expression occurs shortly before the MHR3 expression during the last larval molt (Zhou et al., 1998b; Langelan et al., 2000). Therefore, it may be one of the 20E-induced proteins involved in regulating MHR3 expression.

Drosophila E75A and DHR3 (a *Drosophila* homolog of MHR3) bind to the sequence A/GGGTCA preceded by an AT-rich region as a monomer (Horner et al., 1995). This element is a typical binding element for the retinoid orphan receptor (RORα) in vertebrates (Giguére et al., 1994). In the MHR3 promoter region, there is a consensus monomeric response element (MRE2) (A/T-AGGTCA) (Harding and Lazar, 1993; Giguére et al., 1994; Horner et al., 1995) near EcRE4, and both E75A and MHR3 bound to MRE2 (Fig. 8). The expression of E75A suppressed the activation of the

MHR3 promoter, whereas the removal of the region containing this element abolished this suppressive action (Fig. 7). Therefore, in *Manduca* epidermis, the presence of E75A early in the ecdysteroid rise likely prevents the immediate induction of MHR3 when the ecdysteroid titer increases beyond its threshold for induction (Fig. 9). Normally, E75A is present only for a short time (Segraves and Hogness, 1990; Zhou et al., 1998b) so when E75A disappears, MHR3 will be induced. Thus, this interplay among the ecdysteroid-induced transcription factors as well as the ecdysteroid titer itself is critical to the cascade of appearance of these transcription factors.

MHR3 itself also binds to the same MRE and inhibits its own induction (Figs. 7–9). This negative autoregulatory action thus accounts for the observation that when *Manduca* larval epidermis is exposed to 20E in vitro, MHR3 mRNA first increases, then plateaus, followed by a decrease (Palli et al., 1992). The decline was prevented by the presence of a protein synthesis inhibitor.

A *Bombyx* homolog of MHR3, BmHR3, is not only activated by 20E (Eystathioya et al., 2001), but also activates a synthetic promoter which contains four copies of the RORα response element. But *Bombyx* E75A represses this BmHR3-dependent transactivation of the promoter (Swevers et al., 2002). This suppression by E75A is due to the competition with MHR3 at the DNA binding site, and competition for a coactivator binding site located at the C-terminus of BmHR3. We do not know the inhibitory mechanisms of E75A in our case. Further studies are necessary to understand how the binding of E75A to MRE2 interacts with other factors to suppress the MHR3 promoter.

During the last larval molt in *Manduca*, when the ecdysteroid titer begins to increase, the EcR-B1 mRNA increases in the epidermis, and EcR-A and USP-2 mRNAs are also induced with the high titer (Jindra et al., 1996; Hiruma et al., 1999). USP-1 mRNA declines in response to high ecdysone titer in the presence of JH (Hiruma et al., 1999). These observations in the epidermis do not fit perfectly to the results in the GV1 cell line, suggesting that the regulation of MHR3 gene expression in the epidermal cells may involve other factors that the GV1 cells do not have. When the ecdysone titer begins increasing, E75A mRNA increases but declines later in response to the high ecdysone titer (Zhou et al., 1998b). In contrast, MHR3 mRNA does not increase until the ecdysone titer becomes high (Langelan et al., 2000) when preliminary immunocytochemical studies with an antibody to the common region of E75 show that protein levels are low (Nelson and Riddiford, unpublished). Previous studies with a monoclonal antibody to E75A that showed high levels at this time (Riddiford et al., 2003) are now thought to be artifactual because this antibody does not recognize E75A protein expressed in GV1 cells transfected with pIE1^{hr}/E75A (Nelson, Hiruma, and Riddiford, unpublished). Therefore, part of the timing of MHR3 expression in the epidermis is likely determined by the early suppressive action of E75A on the MHR3 promoter shown in this study. There appear to be other factors in this 20E-induced regulation because an ecdysone-inducible protein(s) is also required for the full activation of the MHR3 gene (Fig. 9). When both USP-2 and EcR-A increase, these factors compete with the EcR-B1/USP-1 complex at EcRE1, so that the activation of MHR3 declines. The switch of USP isoforms during this stage is important to coordinate the expression of MHR3. MHR3 itself and the declining ecdysteroid titer also facilitate the suppression of MHR3.

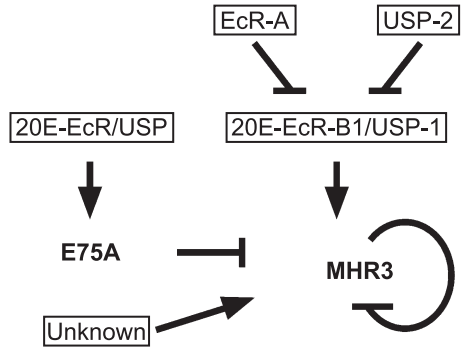

Fig. 9. Summary of the roles of the ecdysone receptor (EcR) and Ultraspiracle (USP) isoforms, and of the transcription factors, E75A and MHR3, on the regulation of MHR3 by 20E.

Acknowledgments

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